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Establishment of *Eucalyptus grandis* W. Hill ex Maiden *in vitro* using commercial products for seed treatment

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Given the current demand for timber from forest species, there is a need to develop new strategies for the mass propagation of eucalyptus. Therefore, this study aimed to determine the effect of different doses of Standak Top® and CoMo Raiz® on the establishment of *Eucalyptus grandis* W. Hill ex Maiden from seeds *in vitro*. The experimental design was completely randomized in a 4 × 3 + 1 factorial arrangement (four concentrations: 1, 2, 3, and 4 mL L^{-1} of the commercial products Standak Top®, CoMo Raiz®, a mixture of both, and a control treatment) with 20 replicates. The following characteristics were evaluated: Contamination level, shoot length, and the number of leaves. No contamination was observed for the different doses of Standak Top®. Additions of 1 to 3 mL L^{-1} of Standak Top® to the culture media yielded the greatest shoot length, and the additions of 2 to 3 mL L^{-1} yielded the greatest number of leaves. Mixing Standak Top® and CoMo Raiz® did not significantly enhance the measured characteristics.

Key words: Standak Top®, CoMo Raiz®, tissue culture, asepsis, culture media.

INTRODUCTION

In Brazil, eucalyptus plants are used primarily as raw material for industries that produce products such as pulp and paper, vegetable coal, poles, fences, and lumber. The production of eucalyptus plants is performed mainly through cloning, which ensures a full retention of the characteristics of selected elite plants and the establishment of uniform high-productivity plots that are disease resistant (Alfenas et al., 2004). Among the various cloning methods, *in vitro* propagation has been successfully used and has been shown to be capable of producing large quantities of new plants from a single explant in a short period of time. Periodic *in vitro* subcultures can be used to reduce the time needed for plantlets to become available (Girijashankar, 2012). In this context, micropropagation is a viable option for *Eucalyptus* sp. propagation due to its advantages over other methods, including a higher multiplication rate, less required physical space, an absence of contaminants and diseases during *in vitro* culture, and more effective control of the factors that are involved (Pinto et al., 2013). In addition to the previously mentioned advantages, Assis and Mafia (2007) suggested using eucalyptus

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micropropagation to rejuvenate selected clones of species and hybrids that have a high commercial value but are difficult to root. Dutra et al. (2009) claimed that some companies use the micropropagated seedlings as an option to obtain better plant material or to supply clonal mini or microgardens. However, forest species are typically difficult to establish *in vitro* due to contaminating agents (Pijut et al., 2012). Several factors must be considered to obtain satisfactory explant decontamination, including the type and age of the material, the type and concentration of the disinfectant, and the duration of explant exposure to the agent (Smith, 2000).

The fungicides most commonly used for *in vitro* disinfection are azoxystrobin, triadimenol, boscalid, pyraclostrobin, carbendazim, tetraconazole, tebuconazole, epoxiconazole + pyraclostrobin, and epoxiconazole. Fungicide concentrations range from 0.1 to 1000 µg of active ingredient (a.i.) mL L^{-1}. The most commonly used antibiotics are ampicillin, chloramphenicol, streptomycin, and tetracycline. Antibiotic concentrations range from 32 to 256 mg L^{-1} (Pereira et al., 2009). Sodium hypochlorite (5%) has been commonly recommended for disinfection to control contamination of the seeds by fungi and bacteria (Rocha et al., 2009; Hariprasad et al., 2013). The same procedure was also recommended by Brondani et al. (2009). Corrêa et al. (2005) suggested using 0.5% active chlorine to disinfect explants because this is a key factor for the remaining steps of *in vitro* establishment.

In vitro studies to evaluate the disinfection efficacy of commercial products, like Standak Top® and CoMo Raiz®, are of interest in order to reduce the production cost and the negative environmental impacts of their use on plant propagation research. Standak Top® is used for seed treatment which contains fipronil, thiophanate-methyl and pyraclostrobin. The manufacturers claim that this formulation is widely used due to its proven phytosanitary action against fungi (protective and anti-sporulating effect). Moreover, its formula acts on cellular respiration, on mitochondria, and on cytochrome Bc_1, transiently interfering with the electron transport chain and, consequently, enhancing CO_2 utilization. Additionally, this product reduces energy expenditure, which results in a greater accumulation of carbohydrates (increased net photosynthesis), increased nitrate reductase activity and chlorophyll content, reduced stress associated with decreased ethylene synthesis, and greater foliar longevity (Balba, 2007). CoMo Raiz® is also used to treat seeds and contains the micronutrients cobalt and molybdenum as well as the growth regulator gibberellin. CoMo Raiz® is used in many cultures because it stimulates germination, rooting and plant growth. The possible effects induced from both commercial products can improve the seedling performance under *in vitro* propagation conditions. This study aimed to determine the effect of different doses of Standak Top® and CoMo Raiz® on the establishment of *Eucalyptus grandis* W. Hill ex Maiden from seeds *in vitro*.

MATERIALS AND METHODS

The plant material used for the *in vitro* propagation of *E. grandis* W. Hill ex Maiden were seeds collected in 2010 and provided by the "Cooperativa Agroindustrial dos Produtores Rurais do Sudoeste Goiano", Brazil. The experiments were carried out at the Plant Tissue Culture Laboratory of the "Instituto Federal Goiano – Câmpus Rio Verde", Goiás, Brazil.

Selected seeds were submersed in running water for 10 min followed by immersion in 70% alcohol for 30 s and then 100% bleach with three drops of 80% Tween for 20 min. The seeds were then washed three times in autoclaved distilled water under a laminar flow hood. These procedures were applied to avoid the *in vitro* contaminants. The seeds were cultured in test tubes (25 x 150 mm) that contained 20 mL of MS culture medium (Murashige and Skoog, 1962) with half the original concentration of salts. The medium was prepared with 3.5 g L^{-1} of agar, 30 g L^{-1} of sucrose, and the commercial products Standak Top® and CoMo Raiz®. The pH of the medium was adjusted to 5.7 ± 0.3 before autoclaving. The experimental treatments included the following doses: 0, 1.0, 2.0, 3.0, and 4.0 mL^{-1}. According to the manufacturers, the recommended dose for treating seeds with Standak Top® and CoMo Raiz® is 2.0 mL L^{-1}. The recommended dose was used as a reference dose to establish the treatments in this study. The treatments were performed according to Table 1.

The inoculated tubes were kept in a growth chamber at 25 ± 2°C and at 45 to 46% relative humidity. Photosynthetic active radiation of 45 to 55 µmol $m^{-2}s^{-1}$ from cool white fluorescent lights was used to produce a photoperiod of 16 h light. Assessments were performed after 30 days of cultivation. The observed characteristics were contamination levels, shoot length, and the number of expanded leaves.

The experimental design was completely randomized in a 4 x 3 + 1 factorial arrangement (four concentrations: 1, 2, 3, and 4 mL L^{-1} of the commercial products Top Standak®, CoMo Raiz®, a mixture of both, and the control treatment) with 20 replicates. Each test tube corresponded to one replicate for a total of 260 experimental units. The experiment was repeated once. The data were tested using ANOVA, and the means were compared according to the Scott-Knott test (5%) using SISVAR software (Ferreira, 2003).

RESULTS

Culture medium containing Standak Top® yielded the best explant response. Specifically, the seedlings showed greater vigor (greater shoot length, number of leaves and intensity of green colour). Moreover, they were well formed without morphological abnormalities, oxidation, or callus formation. Treatment with Standak Top® also yielded greater root formation, leaf number, and shoot length than others (Figure 1 and Table 2). This is the first report to indicate the efficacy of using Standak Top® for protection and vigor improvement of *Eucalyptus* seedlings under *in vitro* propagation. Seedlings cultured in medium containing CoMo Raiz® had less vigor (lower

Table 1. Treatment of *Eucalyptus grandis* seeds with the products Standak Top®, CoMo Raiz®, or a combination of both, and their respective doses.

Treatment	Dose (mL L^{-1})	Product
1	0	Control
2	1.0	Standak Top®
3	2.0	Standak Top®
4	3.0	Standak Top®
5	4.0	Standak Top®
6	1.0	CoMo Raiz®
7	2.0	CoMo Raiz®
8	3.0	CoMo Raiz®
9	4.0	CoMo Raiz®
10	1.0 + 1.0	Standak Top® + CoMo Raiz®
11	2.0 + 2.0	Standak Top® + CoMo Raiz®
12	3.0 + 3.0	Standak Top® + CoMo Raiz®
13	4.0 + 4.0	Standak Top® + CoMo Raiz®

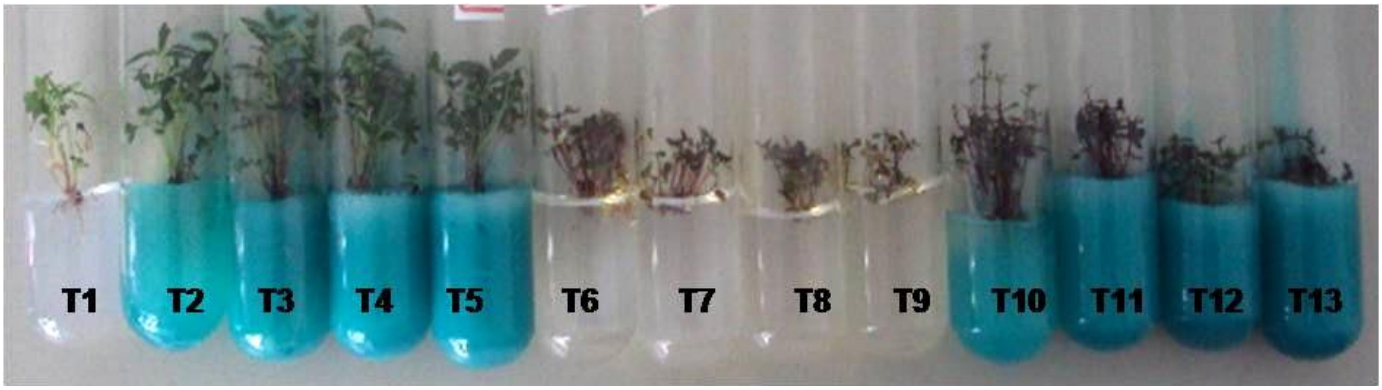

Figure 1. *Eucalyptus grandis* plantlets after 30 days of culture *in vitro* with different doses of Standak Top®, CoMo Raiz®, or a combination of both. Treatments: 1, Control; 2, 1 mL L^{-1} of Standak Top®; 3, 2 mL L^{-1} of Standak Top®; 4, 3 mL L^{-1} of Standak Top®; 5, 4 mL L^{-1} of Standak Top®; 6, 1 mL L^{-1} of CoMo Raiz®; 7, 2 mL L^{-1} of CoMo Raiz®; 8, 3 mL L^{-1} of CoMo Raiz®; 9, 4 mL L^{-1} of CoMo Raiz®; 10, 1.0 + 1.0 mL L^{-1} of Standak Top® + CoMo Raiz®; 11, 2.0 + 2.0 mL L^{-1} of Standak Top® + CoMo Raiz®; 12, 3.0 + 3.0 mL L^{-1} of Standak Top® + CoMo Raiz®; 13, 4.0 + 4.0 mL L^{-1} of Standak Top® + CoMo Raiz®. Bar = 10 mm.

shoot length, fewer leaves, and a brown coloration). Senescence and abscission of some leaves were also observed. The percent of contaminated plantlets receiving the CoMo Raiz® treatment was also greater. Seedlings treated with a combination of Standak Top® and CoMo Raiz® (1.0 + 1.0 mL L^{-1} , 2.0 + 2.0 mL L^{-1}, 3.0 + 3.0 mL L^{-1} and 4.0 + 4.0 mL L^{-1} of Standak Top® + CoMo Raiz®) exhibited explant growth similar to the treatment with CoMo Raiz® alone (1, 2, 3 or 4 mL L^{-1} of CoMo Raiz®) (Figure 1).

There were differences between the treatments and the concentrations of products used. Standak Top® yielded the best results for the contamination index, with an average of 0 to 15% contamination. The treatment containing CoMo Raiz® at a concentration of 4.0 mL L^{-1} also demonstrated an average of 15% contamination, similar to the Standak Top® and the Standak Top® + CoMo Raiz® treatments at concentrations of 2.0, 3.0, and 4.0 mL L^{-1} (Table 2). The highest contamination levels of 40 to 55% were detected in the controls and in the CoMo Raiz® treatments at 1.0 and 3.0 mL L^{-1} (Table 2). The longest shoot lengths of 3.40, 3.45, and 3.38 cm were observed in plantlets that were germinated in culture media containing Standak Top® at concentrations of 1, 2, and 3 mL L^{-1}, respectively. The treatment containing CoMo Raiz® combined with Standak Top® at a concentration of 1 mL each yielded a shoot length of 2.15 cm, which was shorter than the control treatment (2.68 cm)

Table 2. Contamination, shoot length and the number of leaves in *Eucalyptus grandis* as a function of adding Standak Top® and CoMo Raiz® to the germinating culture media.

Treatment	Product	Dosage (mL L^{-1})	Contamination (%)	Length (cm)	Number of leaves
1	Control	0	55^{a}	2.68^{b}	4.90^{c}
2	Standak Top®	1.0	5^{c}	3.40^{a}	6.45^{b}
3	Standak Top®	2.0	0^{c}	3.45^{a}	7.30^{a}
4	Standak Top®	3.0	5^{c}	3.38^{a}	7.75^{a}
5	Standak Top®	4.0	5^{c}	3.00^{b}	6.95^{b}
6	CoMo Raiz®	1.0	50^{a}	1.15^{d}	2.10^{d}
7	CoMo Raiz®	2.0	30^{b}	1.27^{d}	2.70^{d}
8	CoMo Raiz®	3.0	40^{a}	1.42^{d}	2.70^{d}
9	CoMo Raiz®	4.0	15^{c}	1.35^{d}	2.15^{d}
10	Standak Top® + CoMo Raiz®	1.0 + 1.0	25^{b}	2.15^{c}	5.60^{d}
11	Standak Top® + CoMo Raiz®	2.0 + 2.0	5^{c}	1.60^{d}	2.95^{d}
12	Standak Top® + CoMo Raiz®	3.0 + 3.0	15^{c}	1.20^{d}	2.35^{d}
13	Standak Top® + CoMo Raiz®	4.0 + 4.0	5^{c}	1.13^{d}	2.35^{d}

Means within a column followed by the same letter do not differ by the Scott-Knott test (5%).

but longer than other treatments containing both products. Media containing Standak Top® (2 or 3 mL L^{-1}) produced plantlet growth with the most leaves. There was no significant difference in leaf number between the other treatments with different doses of CoMo Raiz® mixed with Standak Top® and the control treatment (Table 2). The average number of leaves was 4.90 for the control treatment. The treatments containing Standak Top® yielded 6.45, 7.30, 7.75, and 6.95 leaves at doses of 1, 2, 3, and 4 mL L^{-1}, respectively.

DISCUSSION

The evaluated characteristics are important variables for assessing the establishment of the studied species *in vitro*. However, from an economic perspective, the most convenient is adding up to 2 mL L^{-1} of Standak Top® per liter of culture medium for tissue culture propagation when using supplementary products that maximize the *in vitro* establishment of *E. grandis* plantlets. For instance, 2 mL L^{-1} is preferable than 3 mL L^{-1} considering the low cost and health perspectives. It should be emphasized that culture medium containing 1 mL L^{-1} of Standak Top® promotes plantlet establishment, less contamination, more leaves, and longer shoots relative to culture medium without Standak Top®.

Seeds contaminated with fungi and bacteria cannot germinate. Therefore, it is necessary to use fungicides in the culture media and/or on the explant. According to Ferreira et al. (2006), using fungicides, such as epoxiconazole, epoxiconazole – pyraclostrobin, and tebuconazole to inhibit *Cylindrocladium candelabrum* in eucalyptus can have phototoxic effects. Fungicides applied at concentrations above the recommended doses may inhibit plant growth (Iqbal et al., 2010) and possibly select for resistant isolates (Leroux, 2003; Verweij et al., 2009). Furthermore, the multiplicity of fungicides' modes of action increases the difficulty of evaluating the risks associated with fungicide use (Yang et al., 2011).

In addition to decontamination, the culture media is another important factor that can influence seedling growth. According to Borges et al. (2011), MS culture medium was appropriate for the multiplication of eucalyptus *in vitro*. In this study, we used MS medium containing half of the original concentration of salts but supplemented with the commercial products Standak Top® and CoMo Raiz®. The establishment percentage of *E. grandis* for treatments with Standak Top® added to the culture media was higher than the percentage for treatments that included CoMo Raiz®.

Fungicides based on strobilurin affect the biochemical and physiological properties of plants. Strobilurins have useful non-fungicidal physiological effects: they improve nitrogen metabolism and also inhibit ethylene biosynthesis. This latter effect is responsible for the greening effect which results in delayed senescence with higher amount of chlorophylls and index of photosynthesis (Häuser-Hahn et al., 2004; Görtz et al., 2008). In this work, the best plant growth may be related to the action of strobilurins which generally increase net photosynthesis and the enzyme nitrate reductase, which is associated with the inhibition of some ethylene synthesis precursors, including aminocyclopropane-1-carboxylic (ACC) synthase and ACC oxidase. Ethylene is a phytohormone involved in chlorophyll degradation (Taiz

and Zeiger, 2010). Thus, the use of Standak Top® is a viable option for maximizing the growth of eucalyptus plantlets.

Conclusions

The use of Standak Top® (2 mL L^{-1}) was effective to increase the growth of *Eucalyptus in vitro* and inhibit fungal and bacterial contamination, considering the low-cost and lower negative environmental impact. Additions of 1 to 3 mL L^{-1} of Standak Top® to the culture media yielded the greatest shoot length, and the additions of 2 to 3 mL L^{-1} yielded the greatest number of leaves. A combination of Standak Top® and CoMo Raiz®, as well as just using CoMo Root® did not produce satisfactory results for the assessed parameters.

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